Developmental Pathways: Sonic Hedgehog–Patched–GLI

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Developmental pathways are networks of genes that act coordinately to establish the body plan. Disruptions of genes in one pathway can have effects in related pathways and may result in serious dysmorphogenesis or cancer. Environmental exposures can be associated with poor pregnancy outcomes, including dysmorphic offspring or children with a variety of diseases. An important goal of environmental science should be reduction of these poor outcomes. This will require an understanding of the genes affected by specific exposures and the consequence of alterations in these genes or their products, which in turn will require an understanding of the pathways critical in development. The ligand Sonic hedgehog, the receptors Patched and Smoothened, and the GLI family of transcription factors represent one such pathway. This pathway illustrates several operating principles important in the consideration of developmental consequences of environmental exposures to toxins. *Key words*: developmental pathways, developmental toxicology, GLI, Patched, Smoothened, Sonic hedgehog. *Environ Health Perspect* 107:167–171 (1999). [Online 20 January 1999]
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A major challenge in environmental health science is the risk of poor developmental outcomes as a consequence of environmental exposure. There is an extensive literature on the effect of toxic environmental exposure on developing systems (*1*), but now the challenge is to determine which specific genes are involved. Evidence from many sources suggests that the number of genes involved is relatively small and it seems that understanding the relationship between environmental exposure, specific mutations, and specific developmental deficits is achievable. This understanding will allow for a rational approach to the refinement of the maternal environment with the goal of improving reproductive outcomes. Our work and that from many other labs (*2–5*) point to threats of chemical exposure to developmental programs in early embryos. Many of these experiments were done with *in vitro* techniques that allowed clear distinction between maternal and embryonic effects. The results clearly indicate that implantation is not a gate through which the embryo must successfully pass and can do so only if it is completely unscathed by genetic damage, but rather it is a threshold over which an embryo, if alive, will cross with accumulated genetic alterations, some environmentally induced (*6*). The sum of these genetic events will determine the final outcome. Deleterious genetic events that occur in critical pathways will result in profound abnormalities. The key to establishing which genes are at risk from which exposures lies in understanding the critical pathways. Information of this sort is now becoming available with remarkable resolution and several operating principles seem to be emerging. First, pathways, not just genes, are conserved from the model systems (*Drosophila* and *Caenorhabditis elegans*, for example) to analogous systems (mouse and rat) and humans. Second, the pathways make use of several levels of regulation: transcriptional, translational, and post-translational. Third, relatively few genes are involved in a combinatorial manner to form complex pathways. Fourth, transcription factors are robustly used to create a pattern for the developing body plan. These principles are well illustrated by the Sonic hedgehog (Shh) pathway and its transcriptional mediator, GLI.

Hedgehog–Patched–GLI Pathway

The Hedgehog (hh)–Patched (Ptc)–GLI pathway (Fig. 1) is critical to several developmental events in a wide range of organisms. It is important in wing development in *Drosophila* (*7,8*), in mesodermal development in vertebrates (*9*), in central nervous system (CNS) patterning in vertebrates (*10,11*), in gastrointestinal (GI) development in mice (*12*) and frogs (*13*), and in axial skeletal patterning in vertebrates (*14,15*). The pathway is active and important in skin and hair follicles (*16,17*), in eye development (*18,19*), and in lung development (*20,21*). In chicken, Indian hedgehog (Ihh) and Shh regulate the targets Ptc and GLI (*22,23*). The Ihh/Shh–Ptc–GLI pathway then regulates BMP2/4 (Dpp in *Drosophila*) (*20,24*). In *Drosophila* hh activates the smoothened (Smo) G-protein-coupled receptor signal transduction pathway through its reaction with the ptc receptor (*25,26*). Activation of this pathway leads to activation of Ci (the *Drosophila* homologue of GLI), which in turn up regulates Ptc, leading to down regulation of Ci (*7*). In mammals Ptc and Smo bind to each other, leading to inactivation of the Smo signal transduction pathway, which is presumed to down regulate GLI (*25*). Shh binds the Ptc receptor and this releases the inhibition of the signal transduction pathway, which is presumed to up regulate GLI activity (*27,28*). GLI3 is thought to down regulate Shh transcriptionally (*29*). The result of misregulation of this pathway and cross-misregulation of GLI2 and GLI3 is abnormal skeletal development in humans (*30*) and mice (*14*) and abnormal GI tract development in mice (*12*) and possibly in humans. CNS defects also result from failure to correctly pattern the ventral floorplate (*10,11,31*).

Disruption of Shh in humans leads to holoprosencephaly, a devastating dysmorphic syndrome with variable expressivity from cyclopia to minor midline fusion defects (*32*). Loss of function mutations in *Ptc*, overexpression of Shh, and activating mutations of GLI cause basal cell carcinoma (BCC), the most common cancer in humans (*17,33–35*) (Table 1). Amplification of *GLI* has been reported in malignant glioma, rhabdomyosarcoma, and osteosarcoma (*36–39*). Disruption of GLI3 causes some forms of Pallister-Hall syndrome (*40*), as well as axial skeletal deformities such as Greig cephalopolysyndactyly and postaxial polydactyly type A (*30,41*). These are very significant diseases in humans: Pallister–Hall syndrome includes hypothalamic hamartoma, postaxial polydactyly, cleft palate, and malformations

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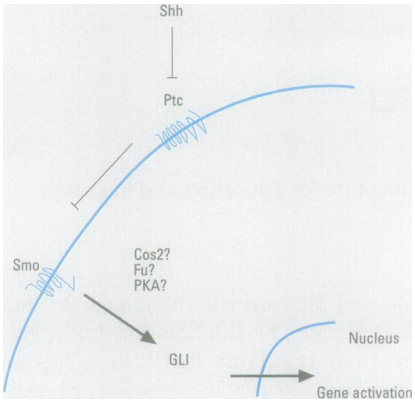

Figure 1. Sonic hedgehog (Shh)–Patched (Ptc)–GLI pathway. The Smoothened (Smo) receptor is a seven-transmembrane-spanning protein that activates GLI. This activation is inhibited by Ptc, a receptor with several transmembrane domains; when the ligand Shh is present Ptc does not inhibit Smo. GLI, when activated, acts as a transcriptional activator. Costal (Cos2) and Fused (Fu) influence the activation of the *Drosophila* homologue Ci and are presumed to affect the vertebrate GLI as well. Protein kinase A (PKA) acts as a negative regulator of Hh signaling in *Drosophila*. The other GLI proteins GLI2 and GLI3 (not shown here) are controlled by the same pathway but their transcriptional activities are less well characterized. GLI3 is thought to down regulate the expression of Shh in vertebrates. In *Drosophila* Ci inhibits the activity of Ptc.

Table 1. Sonic hedgehog–Patched–GLI

Gene	Function of protein	Activation	Human disease	References
Sonic hedgehog	Ligand, inhibits Ptc	Cleavage	Holoprosencephaly	(*32*)
Patched	Transmembrane receptor, inhibits *Smo* in absence of ligand	Shh–NH_3 binding	Basal cell carcinoma, basal cell nevus syndrome	(*17,33–35*)
Smoothened	Transmembrane receptor, activates fused	Inhibited by Ptc in absence of Shh–NH_3	Basal cell carcinoma	(*34*)
Fused	Inhibits PKA, activates GLI	?	?	–
GLI, GLI2, GLI3	Transcriptional factors	$TAF_{II}31$ binding	Greig cephalo-polysyndactyly, Pallister–Hall syndrome, basal cell carcinoma, glioma, rhabdomyo-sarcoma, postaxial polydactyly type A	(*17,30, 36–41*)

Abbreviations: Ptc, patched; Shh, Sonic hedgehog; Shh–NH_3, N terminal fragment of Shh; PKA, protein kinase A.

in a variety of other systems (larynx, heart, and genitourinary system); Greig cephalopolysyndactyly syndrome includes facial abnormalities, extra and deformed fingers and toes, and mental retardation.

Recently, Beachy and co-workers reported that the veratrum alkaloids, long known to cause cyclopia in sheep (*42,43*), inhibit target tissue response to Shh signaling. In chicks, exposure to jervine results in fetal midline fusion defects that are characteristic of holoprosencephaly (*43*). The veratrum alkaloids, and specifically jervine, are structurally like cholesterol and, because the Shh interaction with Ptc requires cleavage of the Shh protein and cholesterol binding, it is tempting to argue that there is a connection between the structure of jervine and its effect. However, simple changes in cholesterol levels do not explain the effect of this teratogen nor does the teratogen inhibit the cleavage. Cooper et al. (*43*) suggest that sterol transport defects in the presence of the teratogen may perturb Ptc function. Although that remains to be seen, it is clear that these compounds and others like them exist in the environment and can exert profound developmental pressure through this pathway.

An increasing exposure risk to ultraviolet radiation from the sun has been ascribed in part to stratospheric ozone depletion and this is an undoubted cause of BCC in humans (*44,45*). Less clear are the genetic causes for this effect. Given that mutations in *Ptc* (*46*), *Shh* (*33–35*), and *GLI* (*17*) all cause BCC, it is likely that environmentally induced genetic damage in this pathway is important in the disease. From what is known about the pathway at this point, the association between mutations in these various genes and a common phenotype is understandable. Loss of function in *Ptc* would have the same effect as gain of function in *GLI* or *Shh*. Clearly, Ptc is a tumor suppressor in the skin, and missense mutations in *Smo* may activate the protein, but it is less clear how mutations in *GLI* transform cells. The skin is sensitive to environmental exposure and the relationship between sun exposure and skin cancer is well established.

There is strong circumstantial evidence for environmental associations in Pallister-Hall syndrome caused by mutations in *GLI3* (*47*). Specific environmental interactions with the Shh–Ptc–GLI pathway have yet to be discovered for postaxial polydactyly, but there is evidence for environmental gene interactions in other axial skeletal malformations (*48*).

At the nexus of the Shh–Ptc pathway are the transcription factors in the GLI family.

The GLI–Kruppel Gene Family: A Zinc Finger Gene Family

GLI is the prototype for the GLI–Kruppel gene family, a gene family characterized by five tandem C_2–H_2 zinc fingers connected by a consensus histidine–cysteine link (*49*). The family is subdivided into *GLI* and Kruppel subclasses on the basis of the specific amino acid sequence in the finger region and the spacing of amino acids between the invariant cysteine and histidine residues (*49*). To date, some members of the gene family in the GLI subgroup include human *GLI*, human *GLI2*, human *GLI3*, human *YY1*, human *ZNF76*, human *ZIC*, murine *gli*, murine *gli2*, murine *gli3*, *C. elegans tra-1*, *Drosophila odd-paired*, and *Drosophila Ci*. Sequence analysis of the coding regions has demonstrated seven areas of similarity among different GLI family proteins, including the zinc finger domain and a carboxy-terminal negatively charged amphipathic alpha-helix, which we have proven is a transcription-activating domain (*50,51*). The functional significance of the other areas of similarity remains uncertain although one of these regions, at the amino terminus, is also conserved in *C. elegans tra-1* and has been suggested to be an inhibitory domain (thus mutations here cause gain of function).

GLI

The 150-kD GLI protein localizes predominantly to the nucleus and binds DNA in a sequence-specific fashion (*52*). Three GLI DNA binding sequences have been identified by DNase footprinting, all of which share the common nine base-pair sequence GACCACCCA (*52*). Crystallographic data indicate that fingers 2 through 5 mediate DNA binding to GACCACCCA (*53*).

Human GLI activates expression of reporter constructs in HeLa cells and with nested deletions transcriptional activity of the protein was shown to require the carboxy-terminal aa 1020–1091. This includes an 18-aa region that is highly similar to the α-helical domain of herpes simplex viral protein 16, including the recognition element for the human TFIID TATA box-binding protein associated factor ($TAF_{II}31$) (*51*). This region is both necessary and sufficient for transactivation. A deletion construct, GLI(-)TAD, abrogates transcriptional activation and lacks this

region yet retains the ability to bind the target DNA sequences, and a deletion construct removing an amino terminal region of the protein enhances transcriptional activation at low GLI concentrations, suggesting this region of the GLI protein is an inhibitory domain (*51*) (Fig. 2).

Human GLI and GLI3 bind identical DNA sequences (*50*). The specific roles of GLI family proteins, GLI, GLI2, and GLI3, in Shh signaling are uncertain. Their functions may be independent, overlapping, or inhibitory. It has been suggested based on the similarity of the binding specificity of GLI and GLI3 that these proteins may assume their specific function through developmentally regulated expression, or through interactions of other nonhomologous domains with additional cellular factors, including specific components of the basal transcriptional machinery and cofactors (*51*). There is evidence to suggest that co-expression of *GLI* and *GLI3* occurs at certain times during development. Co-expression of *GLI* and *GLI3* has been demonstrated in embryonal carcinoma cell lines, an astrocytoma cell line, and a rhabdomyosarcoma cell line (*50*). Second, *GLI* and *GLI3* demonstrate overlapping expression patterns both during certain stages of development and in adult life (*50*). In digit development on day 13, *GLI* and *GLI3* are co-expressed in the mesenchyme of the presumptive digit. Twenty-four hours later, when the phalanges and the interphalangeal joint space appear, *GLI* and *GLI3* have reciprocal expression patterns. *GLI* is expressed in the phalanges and not in the presumptive joint space, while *GLI3* is expressed in the presumptive joint space but not in the phalanges (*54,55*). The transcriptional regulatory mechanisms to account for this are not understood but knockout mice suggest there are both redundant and independent functions for the three *GLI* genes (*14*). Of the three genes only *GLI* can cause neoplastic transformation and is probably the only one regulating proliferation. In the adult human, *GLI3* is expressed in the testis, myometrium, colon, lung, spleen, and placenta; *GLI* is expressed in the normal adult human testis, myometrium, and fallopian tube. Co-expression of *GLI* and *GLI3* suggests that a critical balance between the proteins may be necessary with competition between *GLI*, *GLI3*, and potentially other *GLI* family members for DNA binding sites (*50*).

Although the data suggest that *GLI* and *GLI3* function as transcription factors, the genes regulated by *GLI* and *GLI3 in vivo* remain largely unknown. In *Drosophila*, the homologue Ci regulates *decapentaplegic* (*dpp*) and *wingless* (*wg*), whose vertebrate homologues are the *BMP* and the *Wnt* genes. The only proven target of GLI in vertebrates is HNF3β, a member of the HNF family that represents another network of transcription factors which regulate early development (*56*). It is apparent that interactions in one pathway will have ramifications in other closely related pathways.

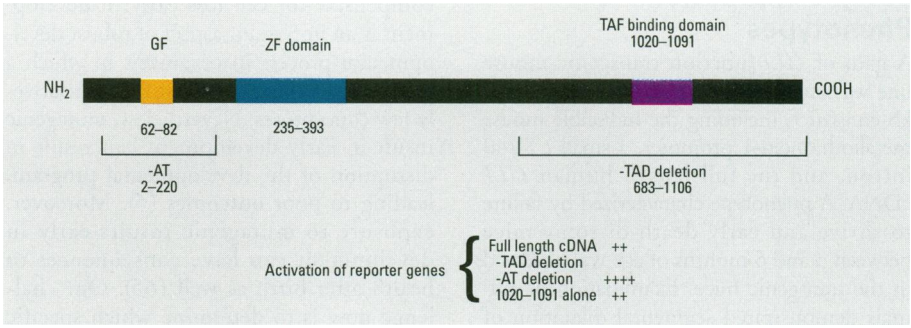

Figure 2. The human GLI protein as a transcriptional activator (numbers refer to aa position).

Homologues of GLI and Conserved Regulation

Drosophila Ci, a homologue of *GLI*, functions as a segment polarity gene during normal *Drosophila* development. *Ci* expression is dependent on a gene cascade including *wg*, engrailed (*en*), *hh*, *ptc*, and *dpp*, and is required for the normal development of the posterior half of each embryonic segment and imaginal discs. *Ci* mutants develop mirror-image duplication of anterior structures in the posterior half of each segment (*7,57*).

C. elegans tra-1, a homologue of *GLI*, functions in a terminal position in the sex determination cascade involving *her-1*, *tra-2*, *tra-3*, *fem-1*, *fem-2*, and *fem-3* in the nematode (*58*). Activity of the gene is required for female somatic differentiation and gain of function mutations feminize the germline (*58*). A post-transcriptional regulation mechanism that is conserved between human and nematodes has been demonstrated (*59*). An element known as the Tra–GLI element (TGE) exists in the 3′ untranslated region of the human *GLI* gene and the *tra-2* gene of *C. elegans*. This element binds to a cellular protein, which shortens the poly A tail and significantly reduces translation of the message into protein. The human gene at any given level of transcription can be down regulated translationally in both *C. elegans* and in mammalian cells, indicating conservation of this regulation mechanism across a vast phylogenetic space. Indeed, the intron–exon boundaries of the region of the gene coding for the zinc finger domain are highly conserved from *C. elegans* to human as well (*60*).

Human Genomic Analysis of GLI

The human *GLI* gene spans 13 kb of genomic DNA and is composed of 12 exons and 11 introns. The five zinc fingers are assembled from exons VII, VIII, IX, and X. The splicing pattern in the *GLI* zinc finger coding region is highly conserved in GLI family members human *GLI2*, *GLI3*, mouse *gli*, and *C. elegans tra-1*, but differs in the zinc finger domain of the GLI family member *YY1*. The full-length mouse *gli* cDNA sequence was determined and showed 85% identity with full-length human *GLI* at the amino acid level but revealed a unique exon in the 5′ untranslated region (*60*).

Primer extension analysis carried out in Tera-1 cells and D259 MG cells identified a major transcription initiation site 157 bp upstream of the GLI translation start site. The region surrounding the major transcription initiation site has a high G–C content but lacks a TATA consensus sequence. The ability of the potential transcriptional regulatory elements in the *GLI* promoter region to drive reporter gene expression was tested by transfection analysis of Tera-1 cells and by microinjection to produce transgenic mice. A 450-bp segment drove expression of a luciferase reporter gene maximally in Tera-1 cells defining a human *GLI* core promoter region. This region contains consensus SP1 and AP2 binding sites. *In vivo* analysis showed that a 1.45-kb upstream segment, including the core promoter region, directed β-galactosidase expression to the CNS at embryonic days 11.5 and 12.5, and to most sites of endochondral ossification at embryonic day 12.5. This pattern driven by the human *GLI* promoter in the mouse embryo demonstrated a comparable pattern to the expression pattern of mouse *gli* during mouse embryonic development, supporting the contention that this region is the *GLI* promoter and that the regulation of *GLI* in the human and mouse is highly conserved (*60*).

Ectopic Expression Phenotypes

A gain of *GLI* function transgenic mouse line was established by microinjecting a 4.5-kb construct, including the inducible mouse metallothionein-1 promoter, a small t SV40 intron, and the full-length human *GLI* cDNA. A phenotype characterized by failure to thrive and early death of some mice between 3 and 6 months of age was observed in the transgenic mice. Examination of animals demonstrated segmental dilatation of the intestine with attenuation of the smooth-muscle layer and decreased density of myenteric plexi dependent on the amount of transgene expression. The colonic epithelium overlying the areas with attenuated smooth muscle was distinctly abnormal, with greatly decreased overall thickness and an absence of goblet cells. The phenotype is similar to that seen more posteriorly when *Hox D13* is knocked out in mice (*61*). Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) showed that the severity of the phenotype was related to the level of expression of the *GLI* transgene (*12*). This model supports a role for *GLI* in growth and may support a role for *GLI* in GI smooth-muscle development, which in the mouse fetus is normally a site of high-level expression during development.

Transgenic mice that ectopically express other members of the pathway have been produced. Because the hh signal is modulated by protein kinase A (PKA), a dominant negative form of PKA was incorporated into transgenic mice. The embryos expressed the transgene in predominately dorsal CNS locations and this resulted in induction of floor plate and motor neuron markers. Misregulation of Shh targets by loss of PKA function can result in the loss of normal CNS development (*62,63*).

Ectopic expression of *GLI* in transgenic mice also results in a misregulation of neuronal development. In the dorsal midbrain and hindbrain *GLI* transgene expression has an effect similar to ectopic expression of *Shh* with an activation of ventral floor plate markers and a suppression of dorsal markers with ectopic formation of dorsal neurons (*64*).

Summary

Developmental strategies range from stereotypical rules of strict ancestral fate where a given cell's potential is determined solely by its parentage, as in *C. elegans*, to highly cell-interactive systems where a given cell's developmental potential is highly dependent on cell-to-cell signaling and microenvironment, as in mammals. An important feature of cell-interactive development is adaptive response to injury. The ability to compensate for cell loss early in development is an important aspect of robust developmental process in organisms in which a great deal of energy is invested in a relatively few conceptuses. Nevertheless, mutagenic insult in early development can result in disruption of the developmental program, leading to poor outcomes (*5*). Moreover, exposure to mutagenic insults early in development can have consequences to health after birth as well (*65*). Our challenge now is to determine which specific genes are altered by given environmental exposures leading to what specific developmental defects. This challenge can be met only with a greater understanding of interconnected developmental pathways that define our body plan and future health.

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